

# Exhibit U

# Detection of Zoonotic Pathogens and Characterization of Novel Viruses Carried by Commensal *Rattus norvegicus* in New York City

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**ABSTRACT** Norway rats (*Rattus norvegicus*) are globally distributed and concentrate in urban environments, where they live and feed in closer proximity to human populations than most other mammals. Despite the potential role of rats as reservoirs of zoonotic diseases, the microbial diversity present in urban rat populations remains unexplored. In this study, we used targeted molecular assays to detect known bacterial, viral, and protozoan human pathogens and unbiased high-throughput sequencing to identify novel viruses related to agents of human disease in commensal Norway rats in New York City. We found that these rats are infected with bacterial pathogens known to cause acute or mild gastroenteritis in people, including atypical enteropathogenic *Escherichia coli*, *Clostridium difficile*, and *Salmonella enterica*, as well as infectious agents that have been associated with undifferentiated febrile illnesses, including *Bartonella* spp., *Streptobacillus moniliformis*, *Leptospira interrogans*, and Seoul hantavirus. We also identified a wide range of known and novel viruses from groups that contain important human pathogens, including sapoviruses, cardioviruses, kobuviruses, parechoviruses, rotaviruses, and hepaciviruses. The two novel hepaciviruses discovered in this study replicate in the liver of Norway rats and may have utility in establishing a small animal model of human hepatitis C virus infection. The results of this study demonstrate the diversity of microbes carried by commensal rodent species and highlight the need for improved pathogen surveillance and disease monitoring in urban environments.

**IMPORTANCE** The observation that most emerging infectious diseases of humans originate in animal reservoirs has led to wide-scale microbial surveillance and discovery programs in wildlife, particularly in the developing world. Strikingly, less attention has been focused on commensal animals like rats, despite their abundance in urban centers and close proximity to human populations. To begin to explore the zoonotic disease risk posed by urban rat populations, we trapped and surveyed Norway rats collected in New York City over a 1-year period. This analysis revealed a striking diversity of known pathogens and novel viruses in our study population, including multiple agents associated with acute gastroenteritis or febrile illnesses in people. Our findings indicate that urban rats are reservoirs for a vast diversity of microbes that may affect human health and indicate a need for increased surveillance and awareness of the disease risks associated with urban rodent infestation.

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Zoonotic pathogens comprise a significant and increasing proportion of all new and emerging human infectious diseases (1, 2). Although zoonotic transmission is influenced by many factors, the frequency of contact between animal reservoirs and the human population appears to be a key element (3). Therefore, the risk of zoonotic transmission is increased by events that act to reduce the geographic or ecological separation between human and animal populations or increase the density and abundance of these populations where they coexist (2, 4). In this context, rapid and continuous urbanization constitutes a significant challenge to human health, as it creates irreversible changes to biodiversity that are driven by varied responses from animal species. In particular, species classified as urban exploiters and urban adapters may exist

in unnaturally large and dense populations within urban environments and have above-average rates of contact with people (5–7). Of these, few species have been as successful at adapting to a peridomestic lifestyle as the Norway rat (*Rattus norvegicus*).

In the urban environment, Norway rats closely cohabit with humans—living inside buildings, feeding on refuse, and coming into contact with many aspects of the food supply (7–9). These characteristics, coupled with high levels of fecundity, growth rates, and population densities, suggest that urban Norway rats may be an important source of zoonotic pathogens (10, 11). Indeed, the Norway rat is a known reservoir of a range of human pathogens, including hantaviruses, *Bartonella* spp., and *Leptospira interrogans*; however, little is known about the microbial diversity pres-

TABLE 1 Numbers of Norway rats and sample types positive for bacterial, protozoan, and viral agents identified by targeted PCR analysis, as well as correlations between presence of pathogen and age of host<sup>a</sup>

Type of microbe	Microbe	Total no. (%) of rats positive ( <i>n</i> = 133)	Sample type(s) (no. of positive samples/no. tested)	<i>P</i> value for correlation with age <sup>b</sup>
Bacteria	<i>Bartonella</i> spp.	33 (25)	Fecal (0/133) Liver (31/133) Serum (0/114) Spleen (31/133)	<0.001 (+)
	<i>C. jejuni</i>	5 (4)	Fecal pellets (5/133)	
	<i>C. difficile</i>	1 (1)	Fecal pellets (1/133)	
	<i>C. perfringens</i>	9 (7)	Fecal pellets (9/133)	
	EPEC (atypical)	50 (38)	Fecal pellets (50/133)	0.023 (–)
	<i>L. interrogans</i>	16 (12)	Brain (0/133) Bladder (1/97) Kidney (12/83) Serum (0/114) Urine (14/36)	<0.001 (+)
	<i>S. enterica</i>	2 (2)	Fecal pellets (2/133)	
	<i>Shigella</i> /EIEC	7 (5)	Fecal pellets (7/133)	
	<i>S. moniliformis</i>	23 (17)	Fecal pellets (1/133) Salivary gland (22/133)	
	<i>Y. enterocolitica</i>	1 (1)	Fecal pellets (1/133)	<0.001 (+)
	SEOV Baxter	8 (6)	Fecal pellets (0/133) Bladder/urine (0/133) Brain (6/133) Kidney (7/133) Liver (3/133) Lung (8/133) Oral swab (3/8) Salivary gland (6/133) Serum (4/8) Spleen (6/133)	
			Fecal pellets (2/133) Lung (0/133)	
Protozoan	<i>C. parvum</i>	2 (2)		

<sup>a</sup> Data are not shown for *C. coli* (fecal samples only), enterohemorrhagic, enterotoxigenic, or enteroaggregative *E. coli* (fecal samples), *L. monocytogenes* (fecal samples), *Rickettsia* spp. (brain, kidney, serum, and spleen samples), hepeviruses (fecal, liver, spleen, and urine samples), *T. gondii* (fecal, brain, liver, and lung samples), *V. vulnificus* (fecal samples), or *Y. pestis* (liver, lung, serum, and spleen samples) as these were not detected in any animal in this study.

<sup>b</sup> +, positive association; –, negative association. No significant associations were detected between the presence of an agent and the sex of the host.

ent in urban rat populations or the risks they may pose to human health (12–16).

As a first step toward understanding the zoonotic disease risk posed by rats in densely urban environments, we assessed the presence and prevalence of known and novel microbes in Norway rats in New York City (NYC). We took the unique approach of using both targeted molecular assays to detect known human pathogens and unbiased high-throughput sequencing (UHTS) to identify novel viruses related to agents of human disease. We quantified the tissue distribution of these novel viruses in the host using molecular methods and, in some cases, identified the site(s) of replication using strand-specific quantitative reverse transcription (RT)-PCR (ssqPCR). Unlike previous urban studies that have primarily relied on serological assays to assess the total prevalence of historic infection, our data provide a snapshot estimate of the current level of infection in the rat population, a parameter more closely related to the risk of zoonotic transmission (12, 17). Furthermore, as previous work has focused on rats found exclusively in outdoor locations, we concentrated our sampling within the built environment, where direct and indirect human-rodent contact is more likely to occur (18).

## RESULTS

**Sample collection.** A total of 133 Norway rats were collected from five sites in NYC. Males (*n* = 72) were trapped slightly more often than females (*n* = 61), and juveniles were trapped more often than any other age category. Of the female rats, 43% were juveniles, 26% were subadults, and 31% were sexually mature adults, whereas 40% of the male rats were juveniles, 33% were subadults, and 26% were sexually mature.

**Targeted molecular analyses.** Specific PCR-based assays were used to screen for the presence of 18 bacterial and 2 protozoan human pathogens (see Table S1 in the supplemental material). None of the samples tested was positive for *Campylobacter coli*, *Listeria monocytogenes*, *Rickettsia* spp., *Toxoplasma gondii*, *Vibrio vulnificus*, or *Yersinia pestis*, despite previous studies documenting most of these in multiple rodent species (15). All other bacterial and protozoan pathogens were detected in at least one animal (Table 1). Three bacterial pathogens were identified in more than 15% of animals: atypical enteropathogenic *Escherichia coli* (EPEC) was the most common (detected in 38% of rats), followed by *Bartonella* spp. (25% of rats) and *Streptobacillus moniliformis* (17% of rats) (Table 1). Phylogenetic analysis of a 327-nucleotide



(nt) region of the *gltA* gene amplified from all infected animals revealed three distinct *Bartonella* species infecting NYC rats (Fig. S1). The most common of these was detected in 76% of *Bartonella*-positive animals and clustered within the *Bartonella tribocorum* group, which has previously been identified in multiple species of *Rattus* in Asia and North America. In addition, sequences 98% similar to those of *Bartonella rochalimae* were recovered from seven rats, and *Bartonella elizabethae* was identified in a single rat (Fig. S2). Infection with *Bartonella* was positively correlated with the age of the rat ( $P < 0.001$ ) but not the sex (Table 1).

Seoul virus (SEOV) was the only virus detected in this study by specific PCR; no hepeviruses or other hantaviruses were identified. Eight rats were positive for SEOV, and there was a positive correlation between the presence of the virus and the age of the rat ( $P < 0.001$ ) but not the sex (Table 1). Quantitative PCR (qPCR) analysis of all samples from infected rats revealed variation in viral RNA levels across tissue types. Lung tissue was the most consistently positive (8/8 samples positive) and had the highest average viral RNA copy number, followed by kidney (7/8 positive), brain, salivary gland, and spleen (6/8 positive) (Fig. 1A). None of the urine samples were positive by any assay, although virus was detected in the oral swabs of the three rats with the highest average viral loads and most widely disseminated infections (Fig. 1A). Our phylogeographic analysis revealed that the SEOV strain sampled in this study, provisionally designated Baxter, is most closely related to the Humber strain (GenBank accession number JX879769) from the United Kingdom (Bayesian posterior probability [BPP] = 1) (Fig. 1B). The introduction of SEOV Baxter into NYC appears to be recent, as our analysis estimated the time to most recent common ancestor (TMRCA) for the Baxter and Humber strains at 3 to 16 years before present (ybp). Complete nucleocapsid (N) and glycoprotein precursor (GPC) gene sequences of SEOV Baxter from the lungs of four rats were 100% and 99.9% identical to each other and 97.1% and 95.5% identical to the N and GPC genes of the Humber strain, further supporting the hypothesis of a recent origin. In addition, both the Humber and Baxter strains appear to have recently emerged from China, sharing a TMRCA of 8 to 35 ybp with the ancestral diversity of Chinese SEOV (Fig. 1B).

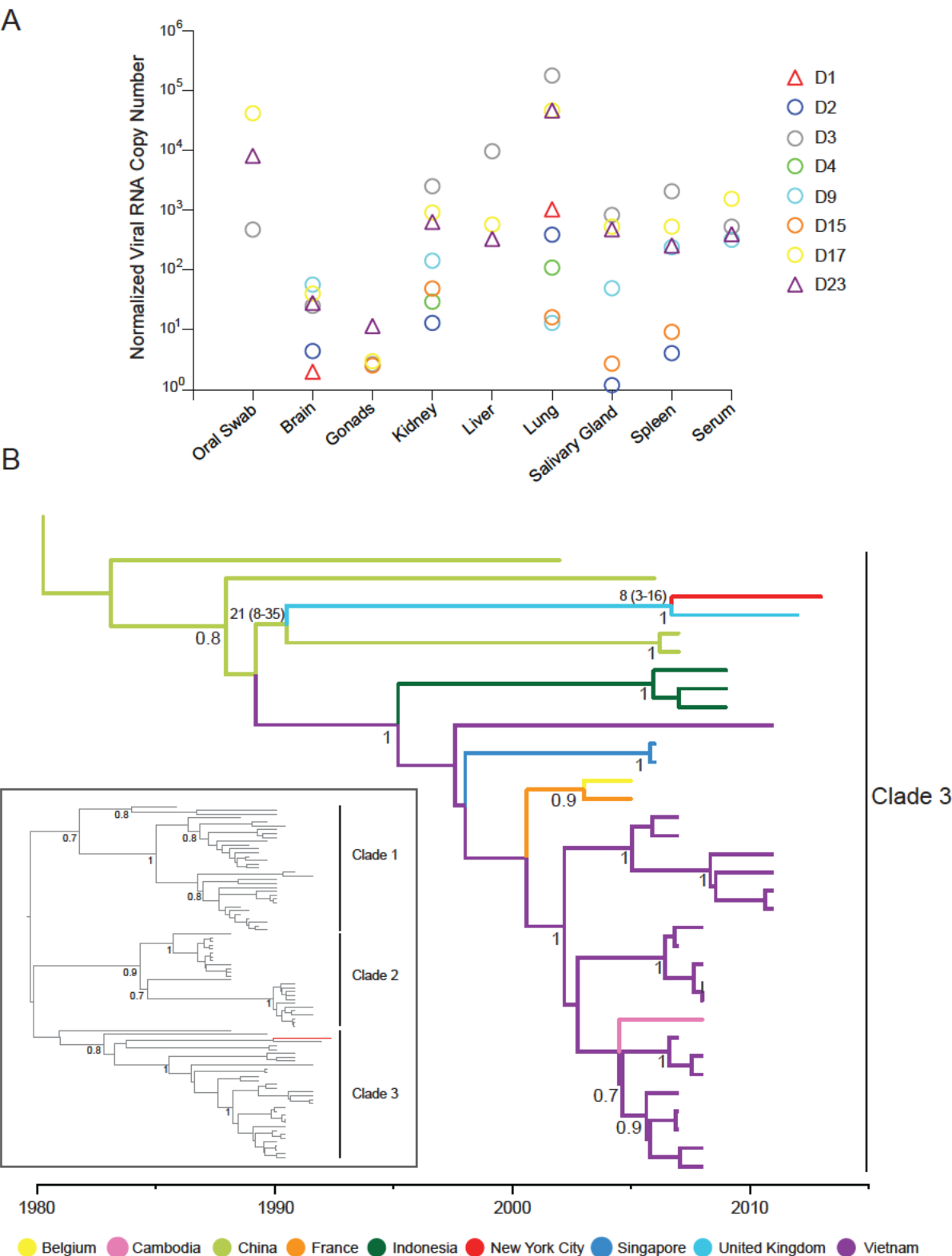
**Viral metagenomic overview.** Serum samples from 114 rats and fecal pellets or rectal swabs from 133 rats were subjected to UHTS in pools of four to six samples. An average of 516,083 reads per pool was generated, with a mean read length of 182 nt (standard deviation, 63 nt) after trimming and filtration. For the purposes of this study, we focused solely on sequences likely to represent mammalian viruses for further analysis. Viruses were grouped into two categories based on percent nucleotide similarity to published sequences, those likely to have been described previously ( $\geq 70\%$  similar) and those likely to be novel ( $< 70\%$  similar) (Table 2). Analysis of the pooled serum samples revealed that viruses in the first category fell into four families or genera (*Anelloviridae*, *Bocavirus*, *Mastadenovirus*, and *Parvovirus*), while those from the pooled fecal samples fell into nine genera (*Bocavirus*, *Calhevirus*, *Cardiovirus*, *Circovirus*, *Hunnivirus*, *Mamastrovirus*, *Mastadenovirus*, *Parvovirus*, and *Rotavirus*). Many of these were  $> 90\%$  similar at the nucleotide level to viruses known to infect Norway rats (e.g., Killham rat virus, rat astrovirus, and infectious diarrhea of infant rats [IDIR] agent [group B rotavirus]) and were not pursued further (Table 2). Viruses from an addi-

tional 13 families or genera that were  $< 70\%$  similar at the nucleotide level to known agents were also identified; five of these were detected in serum samples (*Arterivirus*, *Hepacivirus*, *Orbivirus*, *Pegivirus*, and *Pestivirus*), while the remainder were of fecal origin (*Hepeviridae*-like, *Kobuvirus*, *Parechovirus*, *Phlebovirus*, *Picornavirus*, *Rosavirus*, *Sapovirus*, and unassigned picornaviruses) (Table 2). The presence of all potentially novel viruses was confirmed by PCR using original sample material, and those that fell within groups containing human pathogens were selected for further analysis (Table 3).

**Flaviviruses.** Flaviviruses belonging to the *Hepacivirus*, *Pegivirus*, and *Pestivirus* genera were identified from long contigs in the UHTS data of 16, 15, and 4 pools of serum, respectively (Table 2). All rat sera ( $n = 114$ ) were screened for each virus using a combination of specific and degenerate primer pairs, followed by screening of oral swabs, urine (where available), and fecal samples from infected animals to identify potential routes of transmission (Table 3; see Table S1 in the supplemental material). In total, two novel hepaciviruses (Norway rat hepacivirus 1 [NrHV-1] and NrHV-2), one novel pegivirus (NrPgV), and one novel pestivirus (NrPV) were detected in 36 rats (Table 3). There was a positive correlation between the presence of NrHV-1, NrPgV, and NrPV and the age of the rat ( $P < 0.008$ ) but not the sex. Although 19 animals were infected with only one flavivirus, coinfection was also a common occurrence—10 animals were infected with two flaviviruses, and 6 animals were infected with three or more. We detected viral RNA in the oral swab samples of rats infected with all four flaviviruses, and all but one of the positive swab samples were from adult rats (Table 3). Viral RNA was also detected in the urine and fecal samples from NrHV-1- and NrPgV-infected rats, whereas only urine was positive from NrHV-2- and NrPV-positive animals (Table 3). Although the detection of viral RNA in saliva and excrement does not indicate the presence of intact and infectious viral particles, it does suggest the possibility that transmission of these viruses between animals may occur by inhalation, biting, or ingestion rather than strictly through blood-borne or sexual transmission.

In our phylogenetic analysis of the nonstructural protein 3 (NS3) (data not shown) and NS5B translated amino acid (aa) sequences of these viruses, NrHV-1, NrHV-2, and NrPgV each clustered within clades containing recently described rodent- and bat-borne viruses from North America, Europe, and Africa, along with viruses from other nonhuman mammalian hosts (Fig. 2) (19–21). Despite the existence of related viruses in other, nonurban rodent species, the complete polyproteins of NrHV-1 (3,002 aa), NrHV-2 (2,855 aa), and NrPgV (3,340 aa) were only 23 to 48% (NrHVs) and 26 to 52% (NrPgV) identical at the amino acid level to those of related viruses, suggesting that they are likely distinct and novel species. In our phylogenetic analysis of the *Pestivirus* genus, NrPV fell at the base of the pestivirus clade and appeared to be highly divergent from previously described pestiviruses (Fig. 2). However, a detailed examination of the NrPV genome revealed extensive similarities with the genomes of other pestiviruses. The 5' untranslated region (UTR) of NrPV was of standard length (399 nt) and was predicted to conform to the typical structure of a pestivirus type IV internal ribosomal entry site (IRES) (see Fig. S2 in the supplemental material). The polyprotein sequence of NrPV (3,993 aa) shared a maximum amino acid identity of 60% with other pestivirus polyproteins and was predicted to encode the 12 conserved pestivirus peptides (22).

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**FIG 1** Viral RNA quantification and phylogenetic relationships of SEOV Baxter. (A) Quantification of SEOV Baxter RNA in tissue, oral swab, and serum samples from infected animals collected in this study. Viral RNA copy numbers were calculated per copy of GAPDH for tissue samples or per 1 ml for serum and oral swab samples. (B) MCC tree showing close phylogenetic relationships between SEOV Baxter and viruses from the United Kingdom and China. Estimated times to most recent common ancestor are shown in years above the nodes leading to SEOV Baxter, with the associated 95% highest probability density values in parentheses. Branch colors correspond to countries for which N gene sequences of SEOV were available for analysis, and BPP values of  $\geq 0.7$  are shown beneath the associated nodes.

TABLE 2 Known and novel viruses detected in Norway rat serum or fecal samples by UHTS

Virus type <sup>a</sup>	Sample type	Family or genus	No. of singletons	No. of contigs	Maximum contig length	No. of pools	Most similar species
Known	Serum	<i>Anelloviridae</i>	167	14	1,711	22	Unclassified anellovirus
	Serum	<i>Bocavirus</i> <sup>b</sup>	85	5	2,774	9	Porcine bocavirus
	Serum	<i>Mastadenovirus</i>	62			1	Murine adenovirus A
	Serum	<i>Parvovirus</i>	298	34	2,847	19	Kilham rat virus
	Fecal	<i>Bocavirus</i>	537	40	2,280	16	Porcine bocavirus
	Fecal	<i>Calhevirus</i> <sup>b</sup>	576	71	7,996	10	Calhevirus 1
	Fecal	<i>Cardiovirus</i> <sup>b</sup>	39,798	651	3,938	21	Boone cardiovirus
							Rat theilovirus 1
	Fecal	<i>Circovirus</i>	197	10	3,438	21	Pig stool-associated circular ssDNA virus
							Chicken anemia virus
	Fecal	<i>Hunnivirus</i> <sup>b</sup>	1,297	88	7,511	19	Ovine hungarovirus
	Fecal	<i>Mamastrovirus</i>	75,847	534	3,101	19	Rat astrovirus
							Murine astrovirus
	Fecal	<i>Mastadenovirus</i>	610	79	6,142	18	Murine adenovirus A
	Fecal	<i>Parvovirus</i>	2,237	92	2,710	24	Kilham rat virus
	Fecal	<i>Rotavirus</i> <sup>b</sup>	13			3	IDIR agent
	Fecal	Other <sup>c</sup>	175,647	934	6,252	31	
Novel	Serum	<i>Arterivirus</i> <sup>b</sup>	28	28	2,107	2	Porcine reproductive and respiratory syndrome virus
	Serum	<i>Hepacivirus</i> <sup>b</sup>	39,924	177	8,848	16	Rodent hepacivirus
	Serum	<i>Orbivirus</i> <sup>b</sup>	26			3	African horse sickness virus
							Epizootic hemorrhagic disease virus
	Serum	<i>Pegivirus</i> <sup>b</sup>	3,018	53	2,751	15	Rodent pegivirus
	Serum	<i>Pestivirus</i> <sup>b</sup>	107	13	8,130	4	Bungowannah virus
							Classical swine fever virus
	Fecal	<i>Hepeviridae</i> -like <sup>b</sup>		2	921	1	Hepatitis E virus
							Betatetravirus
	Fecal	<i>Kobuvirus</i> <sup>b</sup>	3,782	206	4,906	19	Mouse kobuvirus
							Aichi virus
	Fecal	<i>Parechovirus</i> <sup>b</sup>	362	25	6,101	5	Ljungan virus
							Sebokele virus
	Fecal	<i>Phlebovirus</i> <sup>b</sup>	1			1	Echarate virus
	Fecal	<i>Picobirnavirus</i>	446	59	2,711	22	Human picobirnavirus
							Otarine picobirnavirus
	Fecal	<i>Picornaviridae</i> <sup>b</sup>	5,223	154	4,444	33	Feline picornavirus
							Bat picornavirus 3
	Fecal	<i>Rosavirus</i> <sup>b</sup>	40,990	369	3,710	20	Rosavirus M-7
	Fecal	<i>Sapovirus</i> <sup>b</sup>	284	19	2,386	13	Human sapovirus
							Porcine sapovirus

<sup>a</sup> Known viruses are defined as having ≥70% similarity and novel viruses as having <70% similarity at the nucleotide level.<sup>b</sup> Presence confirmed by PCR.<sup>c</sup> These included densoviruses, dependoviruses, dicistroviruses, nodaviruses, tobamoviruses, a variety of bacteriophage, and endogenous viral elements (EVEs).

Notably, the N-terminal autoprotease (N<sup>pro</sup>) coding region of NrPV (273 aa) was substantially longer than the 168-aa peptide present in all other pestiviruses and was also highly divergent (maximum amino acid identity of 31%). Despite this extensive sequence divergence, we were able to identify the conserved catalytic residues (i.e., His49 and Cys69) that appear to be necessary for the proteolytic function of N<sup>pro</sup>, although these residues were present at positions His158 and Cys176 of the NrPV N<sup>pro</sup> peptide (22, 23).

**Norway rat hepaciviruses as a model for human hepatitis C virus infection.** NrHV-1 and NrHV-2 are related to human hepatitis C virus (HCV), which is the primary etiologic agent of non-A, non-B viral hepatitis in humans. The identification of related viruses in a species commonly used to model human disease could have medical significance; therefore, we sought to determine if, like HCV, the NrHVs replicate in the liver. ssqPCR was used to measure the relative amounts of the genomic (positive-strand) and replicative (negative-strand RNA intermediate)

forms of NrHV-1 and NrHV-2 across available samples from four NrHV-1-positive rats and both NrHV-2-positive rats (Fig. 2). Variation in positive-strand copy number across tissue types was observed for both NrHV-1 and NrHV-2. Positive-strand viral RNA was most consistently detected in liver tissue and serum by both ssqPCR assays (6/6 animals), and these samples also contained the highest average positive-strand copy numbers across all six rats (Fig. 2). Replicative intermediates (negative-strand viral RNA) were detected exclusively in the liver samples of all six animals tested and ranged from  $9.14 \times 10^2$  to  $1.13 \times 10^5$  RNA copies/250 ng of tissue (Fig. 2). In all livers, the amount of positive-strand RNA was greater than the amount of negative-strand RNA, with the ratio of positive to negative strands ranging from 17:1 to 84:1 in these samples. These findings are consistent with the hepatotropism observed in human HCV infection.

**Picornaviruses.** Analysis of the UHTS data from pooled fecal samples revealed a high diversity of picornaviruses that was comprised of cardioviruses, hunniruses (HuV), kobuviruses (KoV),



TABLE 3 Numbers of Norway rats and of samples positive for viruses identified in UHTS data by specific PCR analysis

Virus	Total no. (%) of rats positive (n = 133)	No. of positive samples/no. tested <sup>a</sup> :														
		Initial screening		Additional screening of indicated sample type in positive animals												
		Fecal	Serum	Bladder	Urine	Fecal	Brain	Heart	Kidney	Liver	Lung	Oral swab	Salivary gland	Serum	Spleen	Intestine
Ro-SaV1	11 (8)	11/133	—	0/9	0/2		0/11	0/11	0/8 <sup>b</sup>	0/11	0/11	—	0/11	0/10 <sup>b</sup>	0/11	—
Ro-SaV2	11 (8)	11/133	—	0/9	0/2		0/11	0/11	0/11	5/11	1/11	0/10	2/11	2/11	5/11	8/10 <sup>b</sup>
NrHV-1	29 (22)	—	27/114	7/11	5/18	1/29	2/5	—	—	29/133	3/5	7/29	3/5		3/5	—
NrHV-2	2 (2)	—	2/114	1/1	1/1	0/2	2/2	—	—	2/133	2/2	1/2	2/2		2/2	—
NrPgV	20 (15)	—	19/114	6/6	7/14	5/20	4/5	5/5	4/5	12/133	5/5	8/19 <sup>b</sup>	5/5		4/5	—
NrPV	6 (5)	—	4/114	—	3/6	0/6	2/6	3/6	1/2 <sup>b</sup>	5/6	4/6	4/6	6/6		6/133	—
NrKoV-1	63 (47)	63/133	—	—	—		1/63	3/63	—	—	3/63	—	—	0/52 <sup>b</sup>	—	—
NrKoV-2	25 (19)	25/133	—	—	—		0/25	0/25	—	—	0/25	—	—	0/19 <sup>b</sup>	—	—
Boone-NYC	37 (28)	37/133	—	—	—		—	—	—	—	—	—	—	—	—	—
NrHuV	21 (16)	4/133	—	—	—		—	—	—	—	—	—	—	—	—	—
RPV	23 (17)	21/133	8/114	—	—		0/5	0/5	0/5	0/5	0/5	—	0/5		0/5	0/2 <sup>b</sup>
MPeV	7 (5)	7/133	—	—	—		0/7	0/7	—	—	2/7	—	—	0/5 <sup>b</sup>	—	—
NrRV	18 (14)	19/133	—	—	—		—	—	—	—	—	—	—	—	—	—
Thera virus-NYC	19 (14)	18/133	—	—	—		—	—	—	—	—	—	—	—	—	—

<sup>a</sup> The proportions of positive samples are given separately for data from the initial screenings used to confirm the UHTS data (performed on fecal and/or serum samples from all animals) and for data from screening of additional sample types from subsets of positive animals (not all sample types were tested for all agents). —, no samples were tested.

<sup>b</sup> Samples were not collected for all positive animals.

parechoviruses (PeV), rosaviruses (RV), and viruses from currently unassigned genera, as well as the picorna-like calhevirus group, which has been predicted to infect insects and was not pursued further (Table 2) (24). To characterize this diversity, fecal samples ( $n = 133$ ) were screened using primers designed from the UHTS data to amplify a highly conserved region of the RNA-dependent RNA polymerase (RdRp) gene of each virus. Phylogenetic analysis of the resulting 385-aa region revealed a total of eight putative species from six genera, of which all but two cardioviruses appeared to be novel (Boone cardiovirus and thera virus) (Fig. 3A; Table 3). A minimum of the complete VP1 gene sequence was obtained from representatives of each virus to aid in taxonomic classification and phylogenetic analysis. As two of the novel viruses, Norway rat hunnivirus (NrHuV) and Norway rat rosavirus (NrRV), fell within well-supported monophyletic clades belonging to genera that do not contain known agents of human disease (the *Hunnivirus* and *Rosavirus* genera, respectively), they were not pursued further in this study.

**Kobuviruses.** Two distinct kobuviruses, provisionally designated Norway rat KoV-1 (NrKoV-1) and NrKoV-2, were detected in the fecal samples of 50% of the rats, although NrKoV-1 was substantially more prevalent (Table 3). Very few other sample types were positive for either KoV by PCR, although three rats were positive for NrKoV-1 in both the heart and lungs (Table 3). The VP1 and RdRp sequences of NrKoV-1 were more than 90% similar to those of a virus previously identified in sewage from Kathmandu (KoV SewKTM), and both of these viruses clustered within a monophyletic clade in the VP1 phylogeny that also included mouse KoV, canine KoV, and aichivirus 1 (bootstrap proportion [BSP] = 94, BPP = 0.99) (Fig. 3B). In contrast, NrKoV-2 was only distantly related to its closest relative, bat KoV TM001k, sharing ~30% aa identity across the VP1 and ~60% aa identity across the RdRp protein sequences (BSP = 94, BPP = 0.94).

**An enterovirus-like picornavirus with an unusual IRES.** A novel picornavirus, provisionally designated rodent picornavirus (RPV), was identified in the fecal and/or serum samples of 23/133 rats in this study (Table 3). RPV formed a distinct lineage within

the clade of the RdRp phylogeny that included the *Sapelovirus* and *Enterovirus* genera, as well as the currently unassigned bat picornaviruses 1, 2, and 3, canine picornavirus, and feline picornavirus (Fig. 3A; see Fig. S3B in the supplemental material). Previous studies have indicated that the feline picornaviruses (and potentially one or more of the bat-associated viruses) should be assigned to a new genus, based on genome organization, G+C content, and the ICTV recommendation that picornaviruses assigned to different genera share no more than 58% aa identity across the polyprotein (22, 25). Therefore, we sought to determine if this proposed new genus should be further expanded to include RPV (Fig. 3A).

The 5' UTR and near-complete coding region of RPV were sequenced from a single fecal sample by overlapping PCR, revealing the characteristic picornavirus gene order 5'-L-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C<sup>pro</sup>-3D<sup>pol</sup>-3' (22). Leader (L) peptides have been identified in the polyproteins of sapeloviruses, as well as the bat, canine, and feline picornaviruses, but are not present in the polyproteins of enteroviruses, suggesting greater similarity between RPV and the former group of viruses. However, as with the L proteins of the bat, canine, and feline picornaviruses, the 64-aa L protein of RPV shared very low amino acid identity with those of other picornaviruses ( $\leq 18\%$ ) and did not appear to contain either the conserved Cys and His residues or the GXCG motif associated with papain- or chymotrypsin-like proteolytic activity, respectively. The RPV polyprotein sequence was also distinct from those of other viruses throughout, sharing only 41 to 43% aa identity with the polyprotein sequences of the bat, canine, and feline picornaviruses and 31 to 37% aa identity with those of select enteroviruses and sapeloviruses. Strikingly, the 5' UTR of RPV showed substantial sequence homology to equivalent genomic regions of members of the *Parechovirus*, *Hunnivirus*, and *Rosavirus* genera and adopted a minimum-energy RNA secondary structure that closely matched the type II IRES found in these other groups (see Fig. S3A in the supplemental material) (26–28). This is interesting because the possession of a type II IRES is unique among viruses within the larger enterovirus/sapelovirus clade. Canine picornavirus, bat picornavirus 3, and all enterovi-

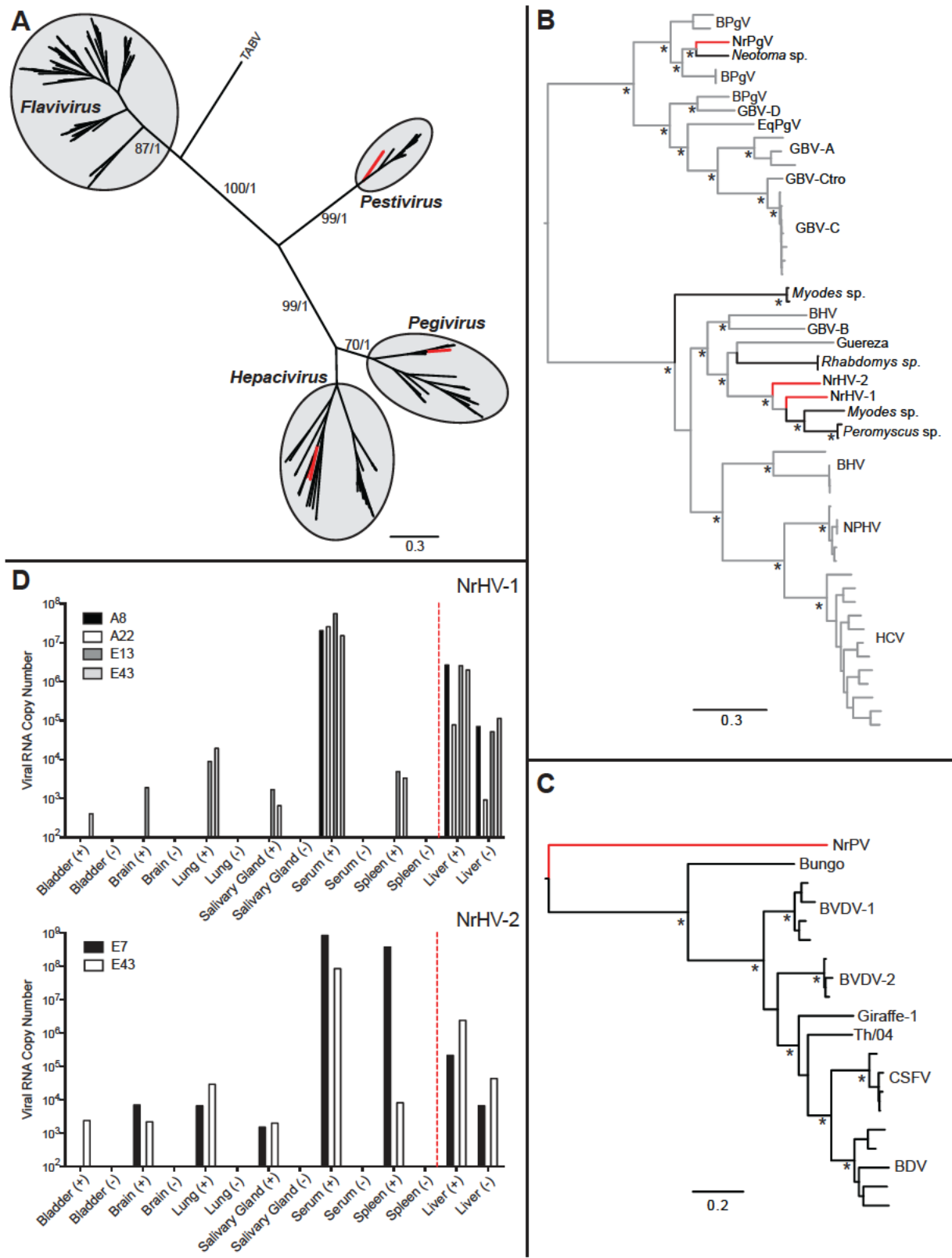


FIG 2 Phylogenetic relationships and strand-specific RNA quantification of the flaviviruses. (A) Unrooted ML tree of a highly conserved region of the NS5B protein (340-aa) of representative members of the *Flaviviridae* family. Grey circles indicate genera, and the four viruses characterized in this study are indicated by red branches. Nodal support is shown beneath associated nodes when both BSP and BPP values are  $\geq 70\%$  in the format BSP/BPP. TABV, Tamana bat virus. The scale bar is in units of substitutions per site. (B) ML tree of the complete NS5B gene of all members of the *Pegivirus* and *Hepacivirus* genera, showing the relative positions of NrPgV, NrHV-1, and NrHV-2 (red branches). For clarity, nodal support values are indicated by an asterisk when both BSP and BPP values

(Continued)



uses possess a type I IRES, whereas a type IV IRES has been identified in the feline picornaviruses, sapeloviruses, and bat picornaviruses 1 and 2. Taken together, these data and the phylogenetic relationships between RPV and other picornaviruses indicate that it may be appropriate to assign RPV to a new genus with a single species, *Rodent picornavirus* (Fig. 3A; see Fig. S3B).

**A new rodent-borne parechovirus.** A novel species of parechovirus, provisionally designated Manhattan parechovirus (MPeV), was identified in 7/133 rats, making it the least prevalent picornavirus detected in this study (Table 3). Screening of additional sample types from these rats revealed two further positive samples, both lung tissue. In the VP1 gene phylogeny, MPeV formed a monophyletic clade with both Ljungan virus and Sebokel virus, which are the only other parechoviruses known to infect rodents and which have also been identified in lung tissue (BSP = 98, BPP = 1) (see Fig. S4 in the supplemental material). MPeV was 40 to 47% identical at the nucleotide level and 35 to 39% identical at the amino acid level to all other parechoviruses across VP1 and 43 to 50% identical at both the nucleotide and amino acid levels across partial sequences (922 nt) of the RdRp gene. In marked contrast to other members of the *Parechovirus* genus, the 552-nt 5' UTR sequence of the MPeV genome possessed no identifiable homology to any picornavirus sequence or known IRES element, and we were unable to derive a convincing secondary structure of the MPeV 5' UTR, despite using a variety of RNA structure prediction methods. These observations provide preliminary evidence that MPeV may contain a previously undescribed IRES type; however, the elucidation of its structure will require comparative analysis with other, as yet undiscovered and similarly unique sequences. Taken together, these data indicate that MPeV may represent a distinct species of parechovirus; however, additional data will be necessary to confirm the tissue tropism, taxonomic status, and genome organization of MPeV (Fig. S4).

**Sapoviruses.** Two distinct and potentially novel sapoviruses (SaV), provisionally designated rodent/Manhattan/2013 sapovirus 1 (Ro-SaV1) and rodent/Manhattan/2013 sapovirus 2 (Ro-SaV2), were each identified in 11 rats (Table 3). Ro-SaV1 was identified exclusively in fecal samples and clustered within a monophyletic clade in the VP1 phylogeny that also included porcine sapovirus (Po-SaV) genogroups GVI and GVII (BSP = 70, BPP = 0.96) (Table 3; Fig. 4). Between animals, the VP1 nucleotide sequences were similar enough for all Ro-SaV1 viruses to be considered a single strain (86 to 90% identical) according to the recently proposed sapovirus genetic classification system (29). However, comparisons of the pairwise nucleotide distances between Ro-SaV1 and Po-SaV GVI and GVII were unable to resolve the taxonomic position of Ro-SaV1, as they fell between the ranges suggested to delineate genotypes and genogroups (0.482 to 0.518) (29). The identification of this virus in a distinct host species suggests that the assignment of Ro-SaV1 to a new genogroup, rather

than a genotype within either Po-SaV GVI or GVII, may be appropriate (Fig. 4).

Unlike Ro-SaV1, Ro-SaV2 was identified in multiple sample types, including intestine, liver, salivary gland, and spleen (Table 3). Ro-SaV2 clustered within a monophyletic clade formed by human sapovirus (Hu-SaV) genogroup GII in the VP1 amino acid phylogeny (BSP = 100, BPP = 1); however, the pairwise nucleotide distances between Ro-SaV2 and Hu-SaV GII.1 to GII.7 (0.384 to 0.428) again indicated an intermediate taxonomic position between a novel genogroup and a new GII genotype (Fig. 4) (29). It is unclear whether Ro-SaV2 should be considered a GII genotype based on the strong monophyly of Ro-SaV2 within the GII clade or whether the presence of the virus in a distinct host species favors the formation of a novel genogroup.

**Microbial burden.** A total of 119/133 rats were positive for at least one microbial agent in our study, with an average burden of 1.6 bacterial agents and 3.1 viruses per rat that did not vary significantly by age group or sex ( $P > 0.05$ ). Only 10 rats were infected with more than two bacterial species, of which eight were female, and no rats were infected with more than four bacterial species (Table 4). In contrast, 53 rats were positive for more than two viral agents, and 13 of these carried more than five viruses (Table 4). As many as nine different viruses or four different bacterial species were identified in the same individual, with a maximum of 11 agents detected in a single rat. Patterns of coinfection between all agents were significantly nonrandom across the complete data set ( $C$  score,  $P = 0.0001$ ); however, the only significantly positive association between any two bacteria occurred between *Bartonella* spp. and *S. moniliformis* ( $P = 0.005$ ). Significantly positive associations were also observed between *Bartonella* spp. and multiple viruses, including NrKoV-1 and NrKoV-2 ( $P < 0.05$ ). Although no significant associations were detected between members of the same viral family, coinfections with multiple flaviviruses and picornaviruses occurred more frequently than expected: NrHV-1 was positively associated with NrKoV-1 and NrKoV-2, and both NrKoV-1 and Boone cardiovirus were positively associated with NrPV ( $P < 0.05$ ).

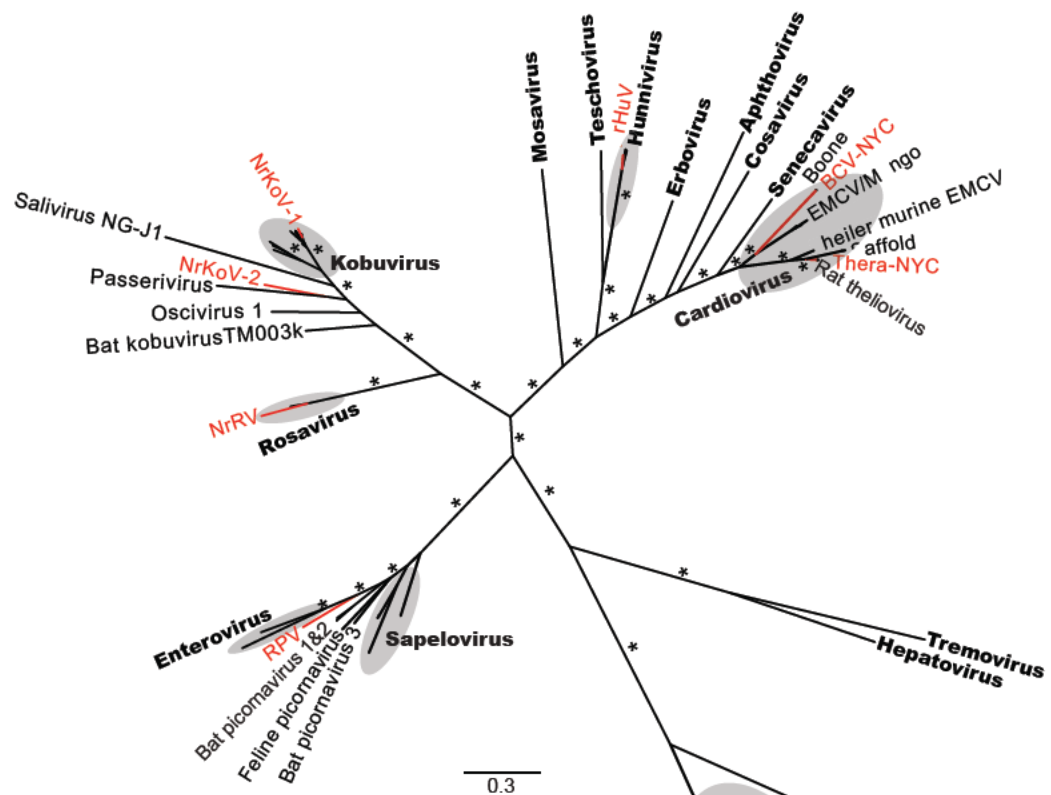
## DISCUSSION

Rodents exist in large populations in urban environments, where they live and feed in closer proximity to people than do most other mammalian species. With continued urbanization, highly successful synanthropic species like the Norway rat are likely to play increasingly important roles in zoonotic disease ecology as the size and complexity of the human-rodent interface increases (30). In this study, we took an important first step toward understanding the risk of zoonotic disease transmission posed by urban rodents by characterizing the microbial diversity and exploring viral infection dynamics in commensal Norway rats in NYC. Notably, we identified nearly 20 distinct mammalian viruses and multiple human bacterial pathogens in our study population, including *Bar-*

### Figure Legend Continued

were  $\geq 70\%$ . The scale bar is in units of substitutions per site. Viruses previously identified in rodents are indicated by the relevant species name (e.g., *Neotoma* sp., *Myodes* sp., *Rhabdomys* sp., *Peromyscus* sp.). BpGV, bat pegivirus; GBV-A to -D, GB viruses A to D or GB virus C troglodytes; EqPgV, equine pegivirus; BHV, bat hepatitis virus; Guereza, guereza hepatitis virus; NPHV, nonprimate hepatitis virus. (C) ML tree of the complete NS5B gene of all members of the *Pestivirus* genus, indicating the basal position of NrPV (red branch). For clarity, nodal support values are indicated by an asterisk when both BSP and BPP values were  $\geq 70\%$ . Bungo, Bungowannah virus; BVDV, bovine viral diarrhoea virus; Giraffe-1, Giraffe-1 pestivirus; Th/04, TH/04\_KhonKaen atypical pestivirus; CSFV, classical swine fever virus; BDV, border disease virus. The scale bar is in units of substitutions per site. (D) ssqPCR quantification of NrHV-1 and NrHV-2 positive- and negative-sense RNA, indicated by + or -, respectively. Viral RNA copy numbers were calculated per 250 ng of tissue or 1 ml of serum.

A



B

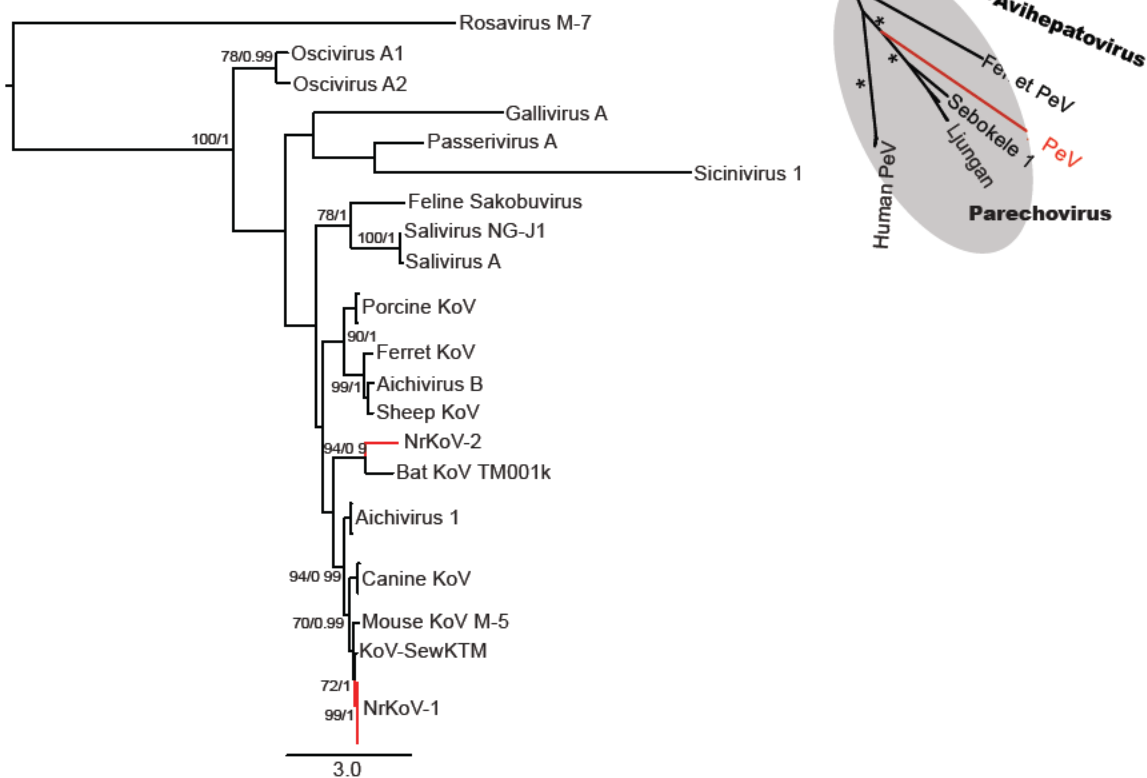


FIG 3 Phylogenetic relationships of the picornaviruses. (A) Unrooted ML tree of a highly conserved, 385-aa region of the RdRp of the eight picornaviruses identified here (red branches) and select representatives of all picornavirus genera (gray circles). For clarity, when BSP and BPP values were both  $\geq 70\%$ , the nodal support is indicated by an asterisk. EMCV, encephalomyocarditis virus; PeV, parechovirus; Sebokel 1, Sebokel virus 1; Ljungan, Ljungan virus. (B) ML tree of the complete VP1 protein of NrKoV-1 and NrKoV-2 (red branches), as well as representatives of the *Kobuvirus* genus. When the BSP and BPP values are both  $\geq 70\%$ , nodal support is shown beneath the associated node in the format BSP/BPP. Scale bar is in substitutions/site.

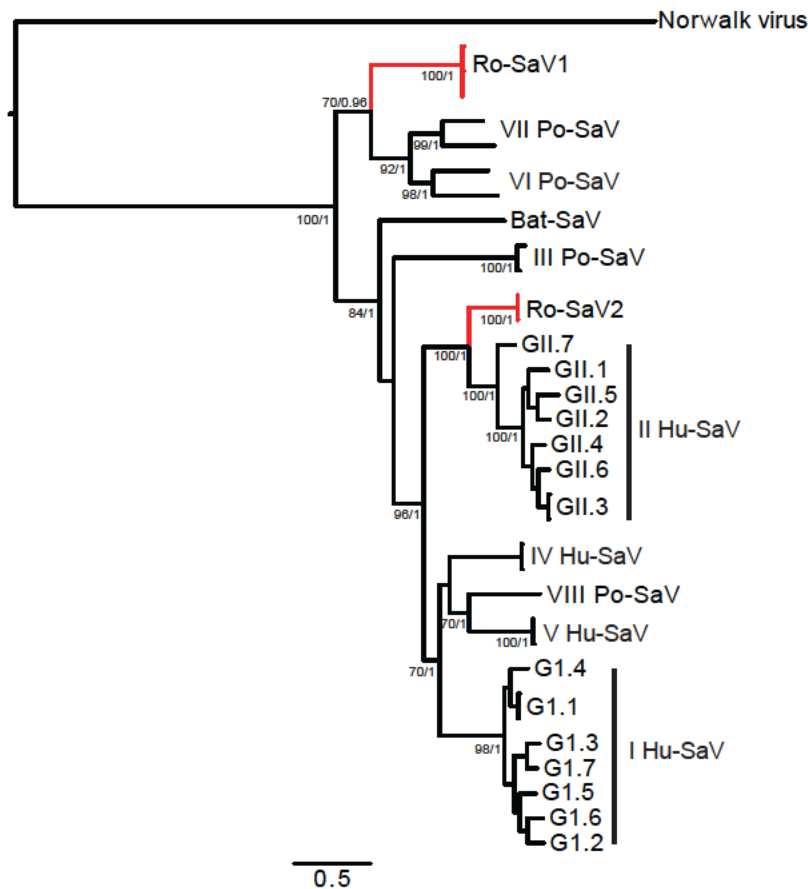


FIG 4 ML tree based on complete VP1 gene sequences of Ro-SaV1 and Ro-SaV2 (red branches) and representatives of the *Sapovirus* genus. Human sapovirus (Hu-SaV) and porcine sapovirus (Po-SaV) genogroups are indicated. When the BSP and BPP values are both  $\geq 70\%$ , nodal support is shown beneath the associated node in the format BSP/BPP. Scale bar is in substitutions/site.

*tonella* spp., *Clostridium* spp., *L. interrogans*, and a plethora of known and novel viruses, including hantaviruses, flaviviruses, and sapoviruses. While a subset of the agents we identified are known

to cause disease in humans, many more are novel viruses whose zoonotic potential cannot be inferred from available data. Although the lack of previous detection of these viruses in human populations suggests that regular zoonotic or sustained transmission is unlikely to be occurring, many rodent-borne pathogens cause only mild or undifferentiated disease in healthy people, and these illnesses are often misdiagnosed and underreported (15, 17, 31–33). It is therefore possible that human infection with some of the agents identified here may already be occurring, and the risk of future zoonotic transmission should not be disregarded. Future work should build on the results of this study and begin to assess the impact of the agents identified here on human health in NYC through continued pathogen surveillance and disease monitoring programs.

**Multiple human pathogens in commensal Norway rats.** In this study, one protozoan and eight bacterial agents commonly associated with mild to severe gastrointestinal disease in humans were identified in rats across multiple sampling sites (Table 1) (14, 15, 34). Widespread prevalence of pathogens, including atypical EPEC and *S. moniliformis* (which can cause severe vomiting in humans), was observed in rats in NYC, along with sporadic infection with several highly pathogenic bacteria, such as *Clostridium difficile* and *Salmonella enterica*. Norway rats, though known carriers of all but one of these agents (*Clostridium perfringens*), are not considered to be a significant factor contributing to the esti-

TABLE 4 Bacterial and viral coinfections identified in the Norway rats in this study<sup>b</sup>

No. of viral agents	Total % of rats positive	No. of bacterial agents						Combined
		0	1	2	3	4		
		31%	37%	25%	6%	2%		69%
0	23%	14	11 <sup>a</sup>	4	1	0		
1	20%	8	12	8	0	0		
2	18%	9	7	5	1	0		
3	14%	4	7 <sup>a</sup>	6	1	1		
4	9%	1	7	1	2	0		
5	7%	3	0	4	2	1		
6	4%	0	1	2	1	0		
7	2%	2	0	1	0	0		
8	2%	0	2	0	0	0		
9	3%	0	2	2	0	0		
Combined	77%							

<sup>a</sup> One animal in this category was also positive for the protozoan *C. parvum*.  
<sup>b</sup> Prevalence is indicated by the intensity of shading. Values indicate the number of rats with each combination of viral and bacterial agents.



mated 2.1 million annual cases of food-borne illness in NYC (14, 15, 35, 36). This may be due to a lack of epidemiological data linking infected rodents to outbreaks of gastroenteritis or food-borne illness in cities around the world, despite evidence of infected rodents in and around farms, residences, markets, and restaurants (15, 37–41). Because rats in NYC and other large urban centers feed on discarded food scraps and household waste, the presence of common enteric pathogens in these rodents is not surprising. However, these same animals also have ready access to locations dedicated to food preparation, storage, and consumption, indicating that rodent infestation in homes and restaurants should be considered a risk factor for the transmission of gastrointestinal disease and food-borne illness.

The rats sampled in this study were also infected with several zoonotic pathogens associated with febrile illnesses, including *Bartonella* spp., *L. interrogans*, and SEOV. To our knowledge, this is the first identification of SEOV in NYC, and the high sequence identity, limited distribution, and recent TMRCA between SEOV Baxter and viruses from the United Kingdom and China suggest a recent introduction into NYC rats (Fig. 1). Widespread serological evidence of exposure to SEOV in rats has been documented in nearby Baltimore since at least 1985, and human infection has been associated with multiple cases of hemorrhagic fever with renal syndrome, chronic renal disease, and asymptomatic infections in Maryland and Los Angeles (12, 17, 42, 43, 79, 80). This indicates that SEOV may have been present in NYC prior to the recent introduction identified here and undergone local extinction and reintroduction. Alternatively, SEOV may exist in multiple small, focal rat populations throughout NYC, of which we have sampled only one. Because the prevalence of rat or human antibody to SEOV has not been assessed in NYC and human infection may be asymptomatic or cause very mild disease, we are currently unable to distinguish between these hypotheses. However, if this virus has only recently been introduced into the NYC rat population, it may represent a potential risk to human health in the city.

Rat populations worldwide are infected with multiple species of *Bartonella*, including the three identified here, *B. elizabethae*, *B. rochalimae*, and *B. tribocorum*. Each of these species has been associated with human disease, including endocarditis or neuroretinitis (*B. elizabethae* only) and febrile illness, although the importance of rodents and their vectors in transmission to humans remains uncertain (15, 44, 45). In NYC, infection with *Bartonella* has not been identified as a significant health threat; however, serosurveys of injection drug users (IDUs) in Central and East Harlem revealed that 47.5% of the study population had a history of exposure to one or more *Bartonella* antigens (46). Although most infections appear to be asymptomatic or result in mild disease (except in rare cases), it is possible that the widespread prevalence of *Bartonella* we observed in NYC rats may impact the health of individuals at risk for opportunistic infections, including IDUs and the immunosuppressed (15, 46–48).

*L. interrogans* is one of the most prevalent and widely distributed zoonotic pathogens in the world, and rodents appear to be the most significant reservoir (14–16). Infection in humans most commonly occurs through the consumption of food or water contaminated with rodent urine but may also result from bites or exposure through the skin and mucous membranes to contaminated soil or water (15, 33). Although we identified *L. interrogans* infection and shedding in only 12% of rats in this study, the prev-

alence of infection in other North American cities has been estimated to be as high as 67% (12, 16). Interestingly, few cases of human disease are reported in the United States each year, which contrasts sharply with the results of human serosurveys that indicate exposure rates as high as 31% in large metropolitan areas, suggesting the potential for both underreporting and misdiagnosis (14, 16, 33). This may be a result of the undifferentiated, self-limiting febrile illness that most commonly occurs as a consequence of human infection and is likely to go unrecognized. The data from our targeted screenings suggest that it will be important to increase diagnostic and surveillance capabilities in NYC to further clarify the risk of human infections from known rodent-borne zoonotic pathogens, including *Bartonella* spp. and *L. interrogans*.

**High viral diversity in commensal Norway rats in NYC.** Despite surveying only 133 rats, we identified a wide diversity of viruses from families and genera that contain important human pathogens, including new genotypes and species of cardioviruses, hepaciviruses, kobuviruses, parechoviruses, and sapoviruses. Of the novel viruses identified here, only NrKoV-1 and Ro-SaV2 are closely related to known human pathogens. NrKoV-1 was the most prevalent agent (bacterial, protozoan, or viral) detected in our study and likely represents a new genotype within the *Aichi virus* species, which appears to have a broad host range that includes humans, canines, deer mice, and rats (49, 50). This suggests that recent and possibly frequent cross-species transmission events have occurred within the species, which may be significant given that Aichi viruses have been associated with acute human gastroenteritis worldwide (50). Strikingly, phylogenetic analysis of Ro-SaV2 revealed that it is more closely related to human viruses than any other mammalian sapovirus described to date. Ro-SaV2 clustered within a clade containing the viruses of human genogroup II, which have been associated with sporadic outbreaks of severe gastroenteritis and food-borne illness (Fig. 4) (51, 52). Together with our identification of an additional, highly divergent sapovirus species (Ro-SaV1), these data suggest that rodents may be an important reservoir of sapovirus diversity globally.

One of the most interesting findings of this study was the uneven representation of viral families in the study population. There was both more diversity (eight distinct species) and a greater abundance (an average of 1.8 viruses per rat) of picornaviruses than of any other viral family, with flaviviruses as the next most frequently encountered family (four distinct viruses with an average of 0.48 per rat). This contrasts sharply with the results of a UHTS study of fecal samples from several wild rodent species, which found evidence of only four picornaviruses (kobuvirus, sapelovirus, mosavirus, and rosavirus) and no flaviviruses (49). Furthermore, the picornavirus diversity we observed is greater than that identified in the virome of any other nonhuman mammal species to date, suggesting that rats may be a significant reservoir for picornaviruses, at least in the urban environment (Fig. 3) (49, 53–56).

Whereas several of the picornaviruses detected in this study fell within genera containing viruses that cause significant human disease, others were highly dissimilar to previously characterized viruses. Both MPeV and RPV were genetically distinct from the most closely related viruses (with RPV possibly representing a novel genus), and each had predicted 5' UTR secondary structures that were unusual or distinctive. The RPV 5' UTR folded into a type II IRES that is unique among viruses within the enterovirus/

sapelovirus lineage but has been identified in parechoviruses, hungaraviruses, and the rodent-borne rosaviruses (26–28). Recombination leading to a modular exchange of at least the IRES element within the 5' UTR is the mostly likely mechanism through which RPV acquired a type II IRES element. A similar process has been proposed for the exchange of type II and type IV IRES elements in other genera, an idea that is favored by the observation that coinfection with multiple picornaviruses appears to be common (28, 53, 57). In contrast, we were unable to identify a secondary structure resembling any known IRES element in the 5' UTR sequence of MPeV *in silico*, despite the sequence similarities between the coding region of this virus and the rodent-borne parechoviruses Sebokele virus and Ljungan virus, which contain type II IRES elements. The discovery of a potentially novel IRES type in MPeV is unusual for picornaviruses, which traditionally contain type I, II, III, and IV IRES elements despite significant variations in other aspects of genome content and organization. Further work will be necessary to elucidate the secondary structure of the 5' UTR of MPeV, using comparative sequence data from other variants of the virus as they become available.

The identification of two novel hepatotropic hepaciviruses in Norway rats is highly significant (Fig. 2). Recent estimates have indicated that up to 3% of the world's population is chronically infected with HCV, and 15 to 30% of those infected will require liver transplants within 20 years (58). However, the lack of a suitable small animal model of HCV infection has meant that many fundamental aspects of HCV infection are unknown or poorly understood, including the determinants of persistence, virulence, pathogenesis, and the host immune response (59). The recent development of a humanized mouse model has the potential to yield important insights into the viral life cycle (60). However, this model is limited because these humanized mice are both immune deficient (*Stat1* knockout) and chimeric (human lymphocytes using mouse lymphoid scaffolding), compromising their utility as a model for natural infection. If it can be demonstrated that the transmission, pathology, and immune responsiveness of rats to NrHV infection closely resemble the etiology of human HCV infection, this system may provide an important and valuable complement to the humanized mouse model. Although NrHVs are only distantly related to HCV, the successful infection of marmosets with HCV-GB virus B (GBV-B) chimeric viruses indicates that future infection of Norway rats with NrHV-HCV recombinants may yield a valuable small-animal-surrogate HCV model system (61). Importantly, the NrHVs identified in this study are the first small-mammal hepaciviruses known to replicate in the liver, a critical component of the HCV life cycle that has yet to be identified in the more closely related equine and canine hepaciviruses (62, 63).

## MATERIALS AND METHODS

**Site selection.** The preliminary nature of this study and the significant complexities involved in trapping rats indoors in NYC necessitated an approach of convenience sampling. An effort was made to target neighborhoods likely to be impacted by the presence of rats, specifically those with high rodent and human density or a high probability of rodent-human interaction. Five sites were selected in midtown and lower Manhattan, comprised of three high-density housing complexes, one very large indoor mixed-use public space (transportation, food service, retail, and commercial), and one small urban park in a densely populated area. The residential sites are on blocks of average density for Manhattan and below-average median income (64). The mixed-use public space is in a

neighborhood notable for an exceptionally high daytime population size and density, and the park was chosen based both on its location (adjacent to the residential sites) and high block density.

**Sample collection.** Norway rats were collected using Tomahawk professional series live traps (Tomahawk Live Trap, Hazelhurst, WI) between September 2012 and June 2013. Rats were euthanized by overanesthesia in isoflurane, followed by bilateral thoracotomy. The animals were measured, weighed, sexed, and bled by cardiac puncture for serum collection. Each rat was assigned to one of three age categories based on body weight, as follows: juvenile (<80 g), subadult (80 to 180 g for females and 80 to 200 g for males), or adult (>180 g for females, >200 g for males) (65). The rats were necropsied, and the following tissues aseptically collected: brain, heart, kidney (83 rats only), liver, lung, inguinal lymph tissue, upper and lower intestine, salivary gland with associated lymph tissue, spleen, gonads (25 rats only), and urine or bladder (when <200  $\mu$ l of urine was available). Oral and rectal swab samples were collected using sterile polyester swabs (Puritan Medical Products Company, Guilford, ME), and fecal pellets were collected when available. All samples were flash-frozen immediately following collection and stored at  $-80^{\circ}\text{C}$ . All procedures described in this study were approved by the Institutional Animal Care and Use Committee at Columbia University (protocol number AC-AAAE6805).

**Targeted molecular analyses.** DNA and RNA were extracted from each tissue and fecal sample using the AllPrep DNA/RNA minikit (Qiagen, Inc.) and from urine or serum using the QIAamp viral RNA minikit (Qiagen, Inc.). The extracted DNA was quantified and diluted to a working concentration of  $\leq 400$  ng/ $\mu$ l. Extracted RNA was quantified, and  $\leq 5$   $\mu$ g used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) and random hexamers. Samples were tested by PCR for 10 bacterial, protozoan, and viral human pathogens previously associated with rodents using novel and previously published PCR assays, including *Bartonella* spp., *L. interrogans*, *Rickettsia* spp., *S. moniliformis*, *Y. pestis*, *Cryptosporidium parvum*, *T. gondii*, hepeviruses, hantaviruses (consensus assay), and SEOV (see Table S1 in the supplemental material). Each assay was performed using a subset of the sample types from each rat, selected to include known sites of replication or shedding (Table 1).

Fecal samples were further analyzed for the presence of the following eight bacterial pathogens commonly associated with human gastrointestinal disease, using PCR-based assays: *C. coli*, *Campylobacter jejuni*, *C. difficile*, *C. perfringens*, *L. monocytogenes*, *S. enterica*, *V. vulnificus*, and *Yersinia enterocolitica* (see Table S1 in the supplemental material). PCR was also used to test for the presence of pathogenic *E. coli*, including enteroinvasive (EIEC, including *Shigella*), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), and enteropathogenic (EPEC) *E. coli* strains, using primers targeting virulence genes (Table S1) (66). In all cases, positive PCR products were confirmed by bidirectional dideoxy sequencing.

Before Ro-SaV2 detection in intestinal samples was attempted, intestines were pretreated to remove fecal contamination by thorough washing with phosphate-buffered saline (PBS). To verify the absence of fecal material in the intestines, a PCR assay for cucumber green mottle mosaic virus (CGMMV) was performed on cDNA from paired fecal and intestinal samples from Ro-SaV2-infected animals. CGMMV was present in 10/11 Ro-SaV2-positive fecal samples and likely originated from the cucumber provided as a water source in the traps. However, all eight intestinal samples that were positive for Ro-SaV2 were negative for CGMMV, suggesting true intestinal infection by Ro-SaV2.

**UHTS.** Serum samples and fecal pellets or rectal swab samples were also extracted, using a viral particle purification procedure, for UHTS. Briefly, each sample was successively passed through 0.45  $\mu$ M and 0.22  $\mu$ M sterile filters (Millipore) to remove bacterial and cellular debris and was treated with nucleases. Samples were lysed in NucliSENS buffer, extracted using the EasyMag platform (bioMérieux), and prepared for sequencing using the Ion Torrent Personal Genome Machine system, following the methods of Kapoor et al. (19). Sequencing was performed on



pools of four to six samples, which were combined at the double-stranded DNA stage. Viral sequences were assembled using the Newbler or miraEST assemblers, and both contigs and unassembled reads were identified by similarity searches using BLASTn and BLASTx against the GenBank nonredundant nucleotide sequence database (67, 68). Viruses related to those known to cause disease in humans were selected for further study and verified by PCR on original (unpooled) sample material with primers derived from the UHTS sequence data. Confirmed positive results were followed by testing of the serum ( $n = 114$ ) or fecal samples ( $n = 133$ ) from remaining animals, and in some cases, subsequent screening of additional sample types from select positive animals (Table 3). One or more positive samples were chosen for further sequencing of phylogenetically relevant genes by overlapping PCR. The 5' UTRs of NrPV, MPeV, and RPV were determined by rapid amplification of cDNA ends (RACE) using the SMARTer RACE cDNA amplification kit (Clontech).

**SEOV Baxter qPCR.** Primers were designed to target a 121-nt region of the N gene (Baxter.qF, 5' CATACCTCAGACGCACAC 3'; Baxter.qR, 5' GGATCCATGTCATCACCG 3'; and Baxter. Probe, 5' -[6-FAM]CCTGGGAAAGGAGGCAGTGGAT[TAMRA]-3' [6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine]). For tissue samples, viral RNA copy numbers were normalized to the quantity of the reference gene encoding glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), whereas the viral RNA copy numbers in serum, oral, and rectal swab samples were reported per ml of serum or PBS wash, respectively (69). qPCR assays were run in duplicate on each sample, and the results were averaged. Samples with an average of  $\leq 2$  normalized copies were considered negative.

**ssqPCR.** For ssqPCR, strand-specific synthetic standards were generated by transcribing positive- and negative-sense RNA *in vitro* from pCRII-TOPO dual promoter vectors (Life Technologies) containing 310 and 594 nt of the NS3 genes of NrHV-1 and NrHV-2, respectively. Positive- and negative-sense RNAs were synthesized from HindIII- or EcoRV-linearized plasmids by transcription from the T7 or SP6 RNA polymerase promoter. *In vitro* transcription was carried out for 2 h at 37°C using the RiboMax large-scale RNA production system (Promega) and 500 ng of linearized plasmid. Plasmid DNA was removed from the synthetic RNA transcripts by treatment with DNase I (Promega) for 30 min, followed by purification with the High Pure RNA purification kit (Roche). Purified RNA transcripts were analyzed on the Agilent 2100 Bioanalyzer, and RNA standards were prepared by serial dilution in human total RNA.

cDNA from both strands was generated using strand-specific primers containing a tag sequence at the 5' end (see Table S2 in the supplemental material) (70). The RNA was preheated at 70°C for 5 min with 10 pmol of specific primer and 1× reverse transcriptase buffer, followed by the addition of a preheated reaction mixture containing 1 mM MnCl<sub>2</sub>, 200 μM each deoxynucleoside triphosphate (dNTP), 40 U RNaseOUT, and 1 U *Tth* DNA polymerase (Promega). The reaction mixtures were incubated at 62°C for 2 min, followed by 65°C for 30 min. The cDNA was incubated with preheated 1× chelate buffer at 98°C for 30 min to inactivate the *Tth* reverse transcriptase before exonuclease I treatment to remove unincorporated RT primers (New England Biolabs). Reaction mixtures lacking RT primer were included to control for self-priming, the strand specificity of each primer was assessed by performing the RT step in the presence of the uncomplementary strand, and reaction mixtures lacking *Tth* DNA polymerase were included to control for plasmid DNA detection.

ssqPCRs were performed using TaqMan universal master mix II with primers, probe, and 2 μl of cDNA under the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 50°C for 20 s, and 72°C for 30 s (see Table S2 in the supplemental material). The specificity of the reaction was monitored by RT and amplification of serial dilutions of the uncomplementary strand. The sensitivities of the ssqPCR assays ranged from  $0.35 \times 10^3$  to  $3.5 \times 10^3$  RNA copies/reaction mixture volume, and nonstrand-specific amplification was not detected until  $3.5 \times 10^7$  viral RNA copies of the uncomplementary strand per reaction mixture volume were present (Table S3).

**Phylogenetic and sequence analyses.** Nucleotide or predicted amino acid sequences were aligned with representative members of the relevant family or genus using MUSCLE in Geneious version 7 (Biomatters Ltd.) and manually adjusted. Maximum-likelihood (ML) and Bayesian Markov chain Monte Carlo (MCMC) phylogenetic trees were constructed for each alignment using RAxML version 8.0 and MrBayes version 3.2, respectively (71, 72). ML trees were inferred using the rapid-search algorithm, either the general time-reversible (GTR) plus gamma model of nucleotide substitution or the Whelan and Goldman (WAG) plus gamma model of amino acid substitution, and 500 bootstrap replicates. MCMC trees were inferred using the substitution models described above, a minimum of 10 million generations with sampling every 10,000 generations and terminated when the standard deviation of split frequencies reached  $<0.01$ . Phylogenetic analysis of *Bartonella* was performed by trimming the *gltA* gene sequences to a 327-nt region (nt positions 801 to 1127) commonly used for taxonomic classification and constructing a neighbor-joining tree using the Hasegawa, Kishino, and Yano (HKY) plus gamma model of nucleotide substitution (13, 73). Phylogenetic analysis of the flaviviruses was performed by first constructing a tree that included representative viruses across the family using a highly conserved region of the NS5B protein (aa 462 to 802 of tick-borne encephalitis virus; GenBank accession number NP\_775511.1), followed by complete NS3 and NS5B amino acid phylogenies constructed separately for the *Pestivirus* and *Hepacivirus*/*Pegivirus* genera. These were rooted based on the relative positions of each genus in the family-level tree.

To estimate the temporal and geographic origin of SEOV Baxter in NYC, phylogeographic analysis of the N gene was performed using the MCMC method available in the BEAST package (version 1.8.0) (74). All available full-length or nearly full-length SEOV N gene sequences with published sampling times were downloaded from GenBank, aligned as described above, and randomly subsampled five times to include a maximum of 10 sequences per country per year. The codon-structured SDR06 model of nucleotide substitution was used along with a relaxed, uncorrelated lognormal molecular clock and a constant population size coalescent prior (best-fit model, data not shown). Two independent MCMC chains were run for 100 million generations each, and convergence of all relevant parameters was assessed using Tracer version 1.5. The runs were combined after removing a 10% burn-in, and the maximum-clade-credibility (MCC) tree, including ancestral location-state reconstructions, was summarized.

Transmembrane domain prediction was performed using TMHMM 2.0, putative N-glycosylation sites were predicted with NetNGlyc 1.0, and the presence of N-terminal signal peptides was predicted using SignalP 4.1, all of which were accessed through the ExPASy web portal (<http://www.expasy.org>). RNA secondary structures were predicted by MFOLD and through homology searching and structural alignment with bases conserved in other pestiviruses for NrPV and with parechoviruses, huniviruses, and rosavirus for RPV (75). RNA structures were initially drawn using PseudoViewer, followed by manual editing (76).

**Statistical analyses.** Potential associations between the presence of a microbial agent and the age and sex of the rat were explored using chi-square tests performed with SPSS version 21 (IBM, Armonk, NY), with associations considered significant at a level of  $\alpha = 0.05$ . Tests for the overall effect of the age category or sex on the number of viruses carried by an individual were conducted using the Kruskal-Wallis test for age and the Wilcoxon test for sex. Patterns of pathogen cooccurrence within a single host were explored using the Fortran software program PAIRS version 1.1, which utilizes a Bayesian approach to detect nonrandom associations between pairs of taxa (77). The *C* score statistic was employed as a measure of pathogen cooccurrence (78).

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the agents sequenced in this study are KJ950830 to KJ951004.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01933-14/-/DCSupplemental>.



Figure S1, PDF file, 0.3 MB.  
Figure S2, PDF file, 0.5 MB.  
Figure S3, PDF file, 0.8 MB.  
Figure S4, PDF file, 0.3 MB.  
Table S1, DOCX file, 0.1 MB.  
Table S2, DOCX file, 0.1 MB.  
Table S3, DOCX file, 0.04 MB.

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